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Interleukin 1 beta induces the formation of nitric oxide by beta-cells purified from rodent islets of Langerhans. Evidence for the beta-cell as a source and site of action of nitric oxide.

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Research Article

Nitric oxide has recently been implicated as the effector molecule that mediates IL-1 beta-induced inhibition of glucose-stimulated insulin secretion and beta-cell specific destruction. The pancreatic islet represents a heterogeneous cell population containing both endocrine cells (beta-[insulin], alpha-]glucagon], gamma[somatostatin], and PP-[polypeptide] secreting cells) and non-endocrine cells (fibroblast, macrophage, endothelial, and dendritic cells). The purpose of this investigation was to determine if the beta-cell, which is selectively destroyed during insulin-dependent diabetes mellitus, is both a source of IL-1 beta-induced nitric oxide production and also a site of action of this free radical. Pretreatment of beta-cells, purified by FACS with IL-1 beta results in a 40% inhibition of glucose-stimulated insulin secretion that is prevented by the nitric oxide synthase inhibitor, NG-monomethyl-L-arginine (NMMA). IL-1 beta induces the formation of nitric oxide by purified beta-cells as evidenced by the accumulation of cGMP, which is blocked by NMMA. IL-1 beta also induces the accumulation of cGMP by the insulinoma cell line Rin-m5F, and both NMMA as well as the protein synthesis inhibitor cycloheximide prevent this cGMP accumulation. Iron-sulfur proteins appear to be intracellular targets of nitric oxide. IL-1 beta induces the formation of an iron-dinitrosyl complex by Rin-m5F cells indicating that nitric oxide mediates the destruction of iron-sulfur clusters of iron containing enzymes. This is further demonstrated by IL-1 beta-induced inhibition of glucose oxidation by purified […]

Interleukin 1β Induces the Formation of Nitric Oxide by β-cells Purified from Rodent Islets of Langerhans

Evidence for the β-cell as a Source and Site of Action of Nitric Oxide

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Abstract

Nitric oxide has recently been implicated as the effector molecule that mediates IL-1β–induced inhibition of glucose-stimulated insulin secretion and β-cell specific destruction. The pancreatic islet represents a heterogeneous cell population containing both endocrine cells (β-[insulin], α-[glucagon], γ-[somatostatin], and PP-[polypeptide] secreting cells) and nonendocrine cells (fibroblast, macrophage, endothelial, and dendritic cells). The purpose of this investigation was to determine if the β-cell, which is selectively destroyed during insulin-dependent diabetes mellitus, is both a source of IL-1β–induced nitric oxide production and also a site of action of this free radical. Pretreatment of β-cells, purified by FACS® with IL-1β results in a 40% inhibition of glucose-stimulated insulin secretion that is prevented by the nitric oxide synthase inhibitor, N^G-monomethyl-L-arginine (NMMA). IL-1β induces the formation of nitric oxide by purified β-cells as evidenced by the accumulation of cGMP, which is blocked by NMMA. IL-1β also induces the accumulation of cGMP by the insulinoma cell line Rin-m5F, and both NMMA as well as the protein synthesis inhibitor cycloheximide prevent this cGMP accumulation. Iron-sulfur proteins appear to be intracellular targets of nitric oxide. IL-1β induces the formation of an iron-dinitrosyl complex by Rin-m5F cells indicating that nitric oxide mediates the destruction of iron-sulfur clusters of iron containing enzymes. This is further demonstrated by IL-1β–induced inhibition of glucose oxidation by purified β-cells, mitochondrial aconitase activity of dispersed islet cells, and mitochondrial aconitase activity of Rin-m5F cells, all of which are prevented by NMMA. IL-1β does not appear to affect FACS®-purified α-cell metabolic activity or intracellular cGMP levels, suggesting that IL-1β does not exert any effect on α-cells. These results demonstrate that the islet β-cell is a source of IL-1β–induced nitric oxide production, and that β-cell mitochondrial iron-sulfur containing enzymes are one site of action of nitric oxide. (*J. Clin. Invest.* 1992. 90:2384–2391.) Key words: aconitase • iron-sulfur protein • insulin-dependent diabetes mellitus • β-cell • cGMP

Introduction

Many lines of evidence indicate that insulin-dependent diabetes mellitus (IDDM)[1] is an autoimmune disease (1, 2). Early stages of IDDM are characterized by infiltration of lymphocytic cells (3), followed by an inhibition of insulin secretion and ultimately selective destruction of β-cells of the pancreatic islet (4, 5). Since macrophage infiltration has been observed early in the disease process, and activated macrophages secrete various cytokines including IL-1β, it has been proposed that IL-1β is a mediator of the autoimmune disease (6). In vitro, IL-1β has been shown to inhibit glucose-stimulated insulin secretion in a concentration and time dependent fashion by isolated islets (6, 7), as well as from β-cells purified by fluorescence activated cell sorting (8). IL-1β has also been shown to induce islet damage and selective destruction of the β-cell after prolonged exposure to this cytokine (4, 5).

Recently, the free radical nitric oxide has been implicated as the effector molecule responsible for the deleterious effects of IL-1β on β-cell function. N^G-monomethyl-L-arginine (NMMA) and N^G-nitro-L-arginine methyl ester, competitive inhibitors of nitric oxide synthase, prevent the inhibitory effects of IL-1β on glucose-stimulated insulin secretion by isolated islets (9, 10). The formation of nitric oxide by islets in response to treatment with IL-1β has been confirmed by electron paramagnetic resonance spectroscopy (EPR). IL-1β has been shown to induce the formation of an axial g = 2.04 EPR-detectable feature by islets that is characteristic of an iron-dithiol-dinitrosyl complex, and the formation of this feature is prevented by NMMA (10). NMMA has also been shown to prevent nonspecific islet cell destruction mediated by activated macrophages (11). These results have established that nitric oxide mediates, in part, the inhibitory effects of IL-1β on glucose stimulated insulin secretion and the cytotoxic effects of activated macrophages on islet cells.

Nitric oxide is formed by the mixed functional oxidation of L-arginine to nitric oxide and L-citruline by nitric oxide synthase (12). At present two isoforms of nitric oxide synthase have been isolated and characterized. One isoform is synthesized constitutively, localized to the cytosol, and is Ca^{2+}- and calmodulin-dependent (13, 14). Synthesis of the other isoform is inducible upon activation by cytokines and lipopolysaccharide (LPS), and is cytosolic and Ca^{2+}-independent (15–17). Nitric oxide produced by these isoforms of nitric oxide syn-

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1. *Abbreviations used in this paper:* EPR, electron paramagnetic resonance spectroscopy; IDDM, insulin-dependent diabetes mellitus; IBMX, isobutyl methylxanthine; NMMA, N^G-monomethyl-L-arginine.

thase has been demonstrated to have different cellular functions. Low levels of nitric oxide released for short periods after receptor or physical stimulation by the constitutive enzyme is believed to function as a cellular signaling molecule (13). Nitric oxide produced after induction of nitric oxide synthase by cytokines or LPS is released for longer periods and in much greater quantities, and appears to function as an effector molecule responsible for the cytotoxic effects of activated macrophages (15). Pancreatic islets appear to contain both isoforms of nitric oxide synthase. Laychock et al. (18) have shown that L-arginine and D-glucose stimulate cGMP formation (nitric oxide is a potent activator of guanylate cyclase, [13]), and that this increase in cGMP is blocked by NMMA. We have demonstrated that IL-1β-induces the formation of the cytokine inducible isoform of nitric oxide synthase by pancreatic islets, and confirmed that nitric oxide mediates IL-1β–induced inhibition of glucose-stimulated insulin secretion by functioning as an effector molecule (10). These studies have suggested a possible signaling role for nitric oxide by the constitutive pathway during glucose-stimulated insulin secretion (18, 19), and an effector role of nitric oxide by the cytokine inducible pathway that mediates the deleterious effects of IL-1β on islet function (10).

The mechanism by which IL-1β inhibits glucose-stimulated insulin secretion by islets is believed to be the result of an impairment in mitochondrial function. Sandler et al. (20, 21) have shown that pretreatment of islets with IL-1β results in an inhibition of glucose oxidation to CO_2, which was demonstrated to reflect an impairment in mitochondrial function. We have recently shown that IL-1β–induced inhibition of glucose oxidation by islets is caused by the formation of nitric oxide, as evidenced by the ability of NMMA to prevent the inhibitory effects of IL-1β on glucose oxidation (22). Furthermore, IL-1β–induced iron-dinitrosyl complex formation by islets suggests that at least one effect of nitric oxide is the destruction of iron-sulfur containing enzymes (10, 22). These results suggest that the cellular mechanism by which IL-1β inhibits insulin secretion may result from the destruction of iron containing enzymes resulting in an impairment in mitochondrial function.

Although advances have been made in elucidating the mechanisms of action of IL-1β on the metabolic functions of the islet, it is currently unknown if IL-1β exerts a direct effect on the β-cell to induce the production of nitric oxide, or if other islet endocrine cells or nonendocrine cells are the source of IL-1β–induced nitric oxide production. Islets contain a heterogeneous population of endocrine cells that include β-cells (60–65%), α-cells (30%), as well as low levels of δ-cells and polypeptide cells (23). In addition, islets also contain nonendocrine cells such as fibroblasts, macrophages, endothelial, and dendritic cells (24). Since islets contain nonendocrine cells that are known to express the cytokine inducible isoform of nitric oxide synthase, and since the β-cell appears to be the only endocrine cell of the islet that is destroyed during the onset of IDDM, the islet cell type in which IL-1β induces the formation of nitric oxide and the effects of nitric oxide on β-cell function were investigated. Evidence is presented which demonstrates that the β-cell is capable of producing nitric oxide in response to IL-1β, and that IL-1β–induced nitric oxide formation results in direct inhibitory effects on β-cell mitochondrial function, thus directly implicating nitric oxide in β-cell specific dysfunction and destruction.

Methods

Materials. Male Sprague-Dawley rats were purchased from Sasco Inc. (O'Fallon, MO). CMRL 1066 tissue culture media were obtained from Gibco Laboratories (Grand Island, NY). Collagenase was obtained from Boehringer-Mannheim (Indianapolis, IN). N^G-monomethyl-arginine acetate was from Calbiochem (San Diego, CA), and recombinant human IL-1β was purchased from Cistron Biotechnology (Pine Brook, NJ). Rin-m5F cells were obtained from the Tissue Culture Support Facility at Washington University School of Medicine (St. Louis, MO). D-[U-^{14}C]Glucose (265 mCi/mmol) was from DuPont New England Nuclear Research Products (Boston, MA). All other chemicals were from commercially available sources.

Purification of β-cells by FACS®. Islets were isolated from 12 male Sprague-Dawley rats (200–300 g) by collagenase digestion as previously described (25), and were cultured overnight (~ 1,800 islets/2.5 ml) in complete CMRL 1066 media (CMRL 1066 containing 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 50 U/ml penicillin, and 50 μg/ml streptomycin) under an atmosphere of 95% air and 5% CO_2. Complete CMRL 1066 media contains 5.5 mM D-glucose. Islets were dispersed into individual cells by treatment with dispase (0.33 mg/ml) in Ca^{2+} and Mg^{2+} free Hanks' solution at 31°C for 15 min (26). The dispersed islet cells were incubated for 45–60 min in complete CMRL 1066 media at 37°C before FACS® cell sorting. Islet cells were purified by the method of Pipeleers et al. (27) using a FACS® 440 (Becton Dickinson, Braintree, MA). The cells were illuminated at 488 nm and emission was monitored at 515–535 nm. The sorting process yields a 90–95% population of β-cells and a 80–85% population of α-cells. The α-cell population also contains some δ-cells and β-cells (26).

Glucose-induced insulin secretion from FACS®-purified β-cells. Purified β-cells were pretreated for 18 h in complete CMRL 1066 or complete CMRL 1066 containing 5 U/ml IL-1β, 0.5 mM NMMA, or IL-1β and NMMA. After pretreatment, the β-cells were washed with Krebs-Ringer bicarbonate buffer (KRB) (25 mM Hepes, 115 mM NaCl, 24 mM $NaHCO_3$, 5 mM KCl, 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, pH 7.4) containing 3 mM D-glucose and 0.5% BSA, counted, and then aliquoted into petri dishes (~ 25,000 cells/dish) containing 1 ml KRB buffer supplemented with either 3 mM or 20 mM D-glucose, 1 mM theophylline, and 0.5% BSA. The cells were incubated for 3 h at 37°C. The cells were transfered to 1.5-ml microfuge tubes, pelleted by centrifugation (2 min 8,000 *g* at room temperature), and insulin was determined on the supernatant by RIA.

Measurement of cGMP levels. FACS®-purified β-cells (~ 10^5 cells/ml) or Rin-m5F cells (~ 10^6 cells/ml) were pretreated for 18 h in 1 ml of complete CMRL 1066 or complete CMRL 1066 containing 5 U/ml IL-1β, 0.5 mM NMMA, cycloheximide, IL-1β, and NMMA, or IL-1β and cycloheximide. After pretreatment, the cells were isolated by centrifugation, washed once in complete CMRL 1066, and incubated three additional hours in complete CMRL 1066 containing 1 mM isobutyl methylxanthine (IBMX) or IBMX and the indicated inhibitors. Samples were treated with the phosphodiesterase inhibitor (IBMX) to prevent the breakdown of cGMP. Also, the inhibitors (NMMA and cycloheximide) were present during the 3-h incubation period to block IL-1β–induced nitric oxide synthase activity, since CMRL 1066 contains 0.3 mM L-arginine, a substrate for nitric oxide synthase. After the 3-h incubation, the cells were isolated by centrifugation, and cGMP was determined using a commercially available RIA kit (DuPont New England Nuclear) as described previously (22).

Glucose oxidation by FACS®-purified β-cells and α-cells. Glucose oxidation was measured on FACS®-purified β-cells and α-cells pretreated for 18 h at 37°C with the indicated concentrations of IL-1β, NMMA, and cycloheximide in 1 ml of complete CMRL 1066 tissue culture media. After pretreatment, the cells were isolated by centrifugation (5 s at 10,000 *g*, microfuge; Beckman Instruments, Inc., Fullerton, CA), washed three times in KRB buffer containing 3 mM D-glucose and 0.5% BSA, and the cells were aliquoted into 1.5-ml microfuge

tubes (~ 150,000 cells/tube). Glucose oxidation was measured as the production of $^{14}CO_2$ from either 3 or 20 mM [U-^{14}C]glucose by FACS®-purified α-cells and β-cells after a 180 min incubation at 37°C (22).

Aconitase activity. Islets (2,400/condition) were isolated and dispersed as stated above. Rin-m5F cells (~ 3×10^7 cells/10 ml of complete CMRL), or dispersed islet cells were cultured for 18 h in the presence or absence of 5 U/ml IL-1β, 0.5 mM NMMA, or IL-1β and NMMA. The cells were isolated by centrifugation (800 *g*, 4°C), and resuspended in 5 ml of buffer (250 mM sucrose, 20 mM Hepes, 10 mM $MgCl_2$, 2 mM KH_2PO_4, and 1 mM EGTA, pH 7.4). The cells were permeabilized with 0.007% digitonin (15–30 min on ice), and then isolated by centrifugation. Permeabilization was monitored by trypan blue exclusion. The cells were lysed by treatment with 0.2% Triton X-100, 30 mM NaCl, and 30 mM Tris-Cl, pH 7.4. The lysate was centrifuged (5000 *g*, 4°C, 15 min), and the supernatant was immediately assayed for aconitase activity. Aconitase activity was followed spectrophotometrically at 340 nm in a solution containing 20 mM citrate, 0.5 mM $NADP^+$, 0.6 mM $MnCl_2$, 50 mM Tris-Cl pH 7.4, 1 U of isocitrate dehydrogenase, and cell extract in a total volume of 1 ml at 21°C. 1 U of aconitase activity is 1 pmol of NADPH formed per minute per milligram of protein.

Electron paramagnetic resonance spectroscopy. EPR spectroscopy was performed at 77 K on Rin-m5F (~ 2×10^7 cells) using a spectrometer (model E-109; Varian Assoc., Inc., Palo Alto, CA), as described previously for islets (10, 22). The microwave power was 1 mW, the modulation frequency was 100 kHz, the modulation amplitude was 12.5 G, and the microwave frequency was 9.105 GHz.

Nitrite determination. Nitrite production by Rin-m5F cells was measured on the supernatant from the same cell cultures used for EPR experiments. The culture supernatant was removed and 100-μl portions were mixed with 100 μl of Griess reagent (28) and 300 μl of distilled H_2O. Nitrite production was determined at an absorbance of 546 nm using a spectrophotometer (DU-6; Beckman Instruments, Inc., Fullerton, CA).

Protein determination. Protein concentration was determined by the method of Bradford (29).

Statistics. Student's *t* test for unpaired data was used to determine statistical significance ($P < 0.05$) and is indicated by *.

Results

IL-1β has previously been shown to inhibit glucose stimulated insulin secretion by purified β-cells (8). To determine if this effect is mediated by nitric oxide, β-cells were pretreated for 18 h with 5 U/ml IL-1β and glucose stimulated insulin secretion was examined. Fig. 1 demonstrates that pretreatment of β-cells with IL-1β results in a 40% inhibition of glucose stimulated insulin secretion. Pretreatment of purified β-cells with NMMA in addition to IL-1β prevents the inhibitory effects of this cytokine on glucose stimulated insulin secretion. Since nitric oxide is a potent activator of guanylate cyclase resulting in the formation of cGMP (13), measurement of cellular cGMP levels represents a sensitive however somewhat nonspecific index for nitric oxide production. Fig. 2 demonstrates that IL-1β induces the accumulation of cGMP in purified β-cells, and that this formation of cGMP is also prevented by pretreatment of β-cells with NMMA in addition to IL-1β. These results indicate that IL-1β-induces the formation of nitric oxide by purified β-cells.

The islet cell type in which IL-1β induces the formation of nitric oxide was investigated further by examining the effects of IL-1β on cGMP accumulation by both β-cells and α-cells isolated by the same FACS® purifications. As demonstrated in Fig. 3, IL-1β induces the accumulation of cGMP in purified

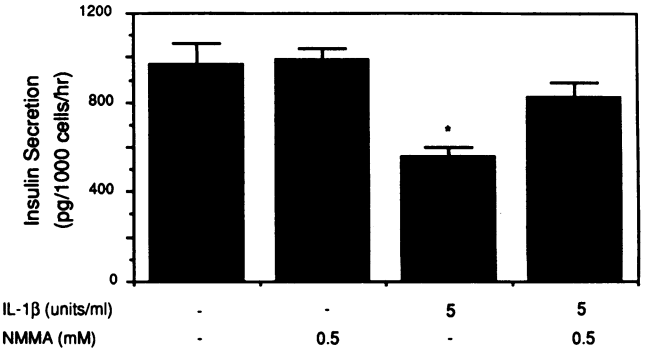

Figure 1. Effects of NMMA and IL-1β on glucose-stimulated insulin secretion by FACS®-purified β-cells. β-cells isolated by FACS® purification were pretreated for 18 h at 37°C in complete CMRL 1066 tissue culture media, or complete CMRL 1066 containing 5 U/ml IL-1β, 0.5 mM NMMA, or both IL-1β and NMMA. After pretreatment, the β-cells were isolated by centrifugation and glucose-stimulated insulin secretion was performed as described under Methods. Results are the mean±SEM of four individual experiments containing at least three replicates in each experiment.

β-cells, but has little effect on cGMP levels of α-cells. A small increase in cGMP accumulation by α-cells in response to IL-1β is observed, however, it is not statistically significant and is consistent with β-cell contamination of the purified α-cell population. In these experiments, the α-cell populations were ~ 80% pure, with the contaminating cells consisting primarily of β-cells. These results indicate that IL-1β does not appear to stimulate nitric oxide formation by α-cells, suggesting that the effects of IL-1β are specific for β-cells of the pancreatic islet. However, it is possible that the α-cell of the pancreatic islet contains extremely low levels of soluble guanylate cyclase, and therefore does not respond to IL-1β.

To further address the question of β-cell specific effects of IL-1β, cGMP accumulation was examined in the insulinoma cell line Rin-m5F. As shown in Fig. 4, IL-1β induces an in-

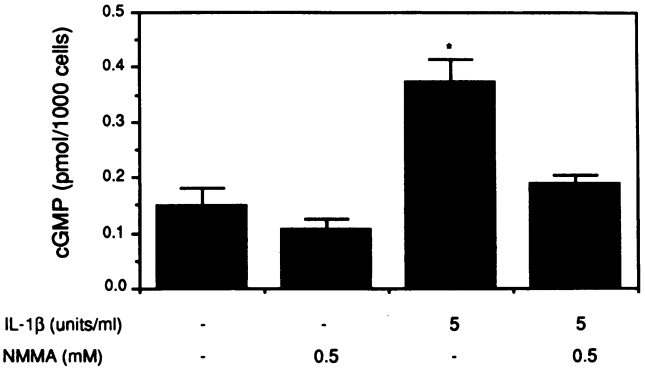

Figure 2. Effects of NMMA on IL-1β–induced cGMP accumulation by FACS®-purified β-cells. β-cells isolated by FACS® purification were pretreated for 18 h at 37°C in complete CMRL 1066 or complete CMRL 1066 containing 5 U/ml IL-1β, 0.5 mM NMMA, or both IL-1β and NMMA. After pretreatment, the β-cells were isolated by centrifugation and treated for three additional hours with 1 mM IBMX or IBMX and 0.5 mM NMMA in complete CMRL 1066. The β-cells were then isolated and cGMP formation was determined by RIA as described in Methods. Results are the mean±SEM of four individual experiments containing three replicates in each experiment.

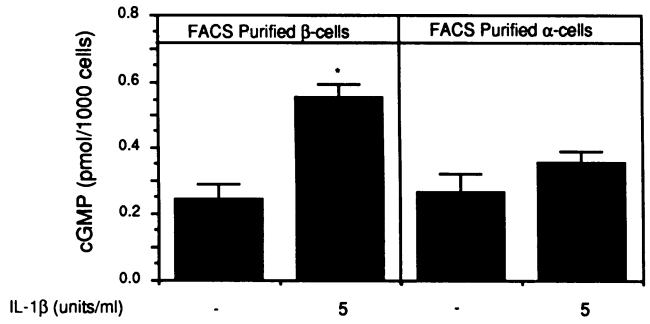

Figure 3. Effects of IL-1β on cGMP accumulation by FACS®-purified β-cells and α-cells. β-cells and α-cells isolated from the same FACS® purification were pretreated for 18 h at 37°C in complete CMRL 1066 tissue culture media with or without 5 U/ml IL-1β. The cells were isolated by centrifugation and treated for three additional hours in complete CMRL 1066 containing 1 mM IBMX, at which time cGMP formation was determined by RIA as described under Methods. Results are the mean±SEM of three individual experiments containing at least three replicates per experiment.

crease in the formation of cGMP by Rin-m5F cells pretreated for 18 h with IL-1β, an effect that is completely prevented by pretreatment of Rin-m5F cells with NMMA in addition to IL-1β. Furthermore, the protein synthesis inhibitor cycloheximide (Fig. 5) also prevents IL-1β–induced accumulation of cGMP by Rin-m5F cells in a dose-dependent manner similar to the effects of cycloheximide on IL-1β–induced accumulation of cGMP by isolated pancreatic islets (22). These results indicate that protein synthesis is required for IL-1β–induced nitric oxide formation by Rin-m5F cells.

To establish that IL-1β induces the formation of nitric oxide by β-cells, EPR spectroscopy was used. Fig. 6 demonstrates that IL-1β induces the formation of a g = 2.04 axial feature by Rin-m5F cells incubated for 18 h with this cytokine, and pretreatment with NMMA in addition to IL-1β significantly blocks the formation of this feature. This feature is characteristic of an iron-dithio-dinitrosyl complex and is believed to arise from the destruction of iron-sulfur clusters by nitric oxide. Furthermore, IL-1β–induced formation of this complex by Rin-m5F cells is prevented by the protein synthesis inhibitor cycloheximide (data not shown). Also shown in Fig. 6 is the simultaneous release of nitrite from the same Rin-m5F cells used for EPR spectroscopy. IL-1β induces the formation of nitrite by Rin-m5F cells, and this accumulation is prevented by NMMA. These results confirm that IL-1β induces the formation of nitric oxide by Rin-m5F cells, and indicate that nitric oxide formation is dependent on both the enzymatic activity of nitric oxide synthase as well as protein synthesis.

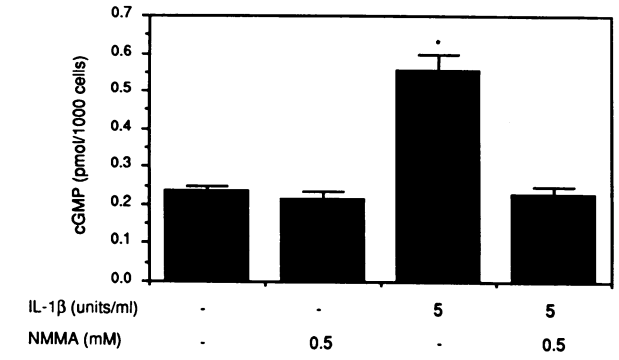

Figure 4. Effects of NMMA on IL-1β–induced cGMP accumulation by Rin-m5F cells. Rin-m5F cells were incubated for 18 h at 37°C in complete CMRL 1066 or complete CMRL 1066 containing 5 U/ml IL-1β, 0.5 mM NMMA or both IL-1β and NMMA. The cells were isolated by centrifugation and incubated for three additional hours in complete CMRL 1066 containing 1 mM IBMX or NMMA in addition to IBMX. The cells were then isolated, and cGMP accumulation was determined by RIA as described under Methods. Results are the mean±SEM of four individual experiments containing four replicates in each experiment.

To determine if IL-1β induces perturbations in β-cell mitochondrial function because of the destruction of iron-sulfur clusters of iron containing enzymes, the oxidation of uniformly labeled [^{14}C]D-glucose to $^{14}CO_2$ by FACS®-purified β-cells was examined. Sandler et al. have demonstrated that the IL-1β–induced inhibition of glucose oxidation by islets is caused by an impairment of islet mitochondrial function (20–21). As shown in Fig. 7, D-glucose (20 mM) produces approximately a threefold increase in the oxidative metabolism of purified β-cells as compared to basal conditions (3 mM glucose), and pretreatment of purified β-cells with IL-1β results in an ~ 80% inhibition of the oxidation of 20 mM D-glucose as compared to untreated control β-cells ($P = 0.056$). Pretreatment of β-cells with NMMA in addition to IL-1β completely prevents the inhibitory effects of IL-1β on glucose oxidation. The effect of IL-1β on the ability of purified α-cells to oxidize glucose to CO_2 was also examined. Pretreatment of purified α-cells for 18 h with IL-1β exerts no effect on the oxidation of 20 mM D-glucose to CO_2 (control α-cells oxidize 4.7±1.8 pmol, while IL-1β treated α-cells oxidize 4.1±0.9 pmol of D-glucose/2,000 cells). These results provide further evidence suggesting that IL-1β–induced metabolic dysfunctions of the islet are caused by specific effects of IL-1β on the β-cell.

IL-1β–induced formation of an iron-nitrosyl complex by Rin-m5F cells (Fig. 6) coupled with IL-1β–induced inhibition of mitochondrial glucose oxidation by purified β-cells (Fig. 7) suggest that nitric oxide mediates the destruction of iron-sulfur clusters of mitochondrial iron containing enzymes. To directly examine this possibility, the effects of IL-1β on mitochondrial

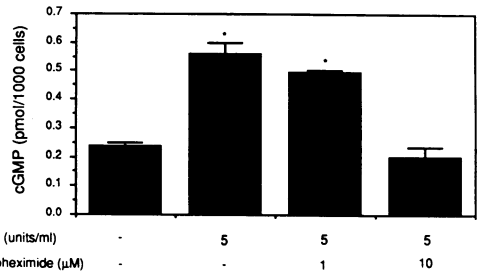

Figure 5. Effects of cycloheximide on IL-1β–induced cGMP accumulation by Rin-m5F cells. Rin-m5F cells were pretreated for 18 h at 37°C in complete CMRL 1066 or complete CMRL 1066 containing 5 U/ml IL-1β, or the indicated concentration of cycloheximide in addition to IL-1β. The cells were isolated by centrifugation and incubated for three additional hours in complete CMRL 1066 containing 1 mM IBMX, at which time the cells were isolated and cGMP formation was determined by RIA as described under Methods. Results are the mean±SEM of four individual experiments.

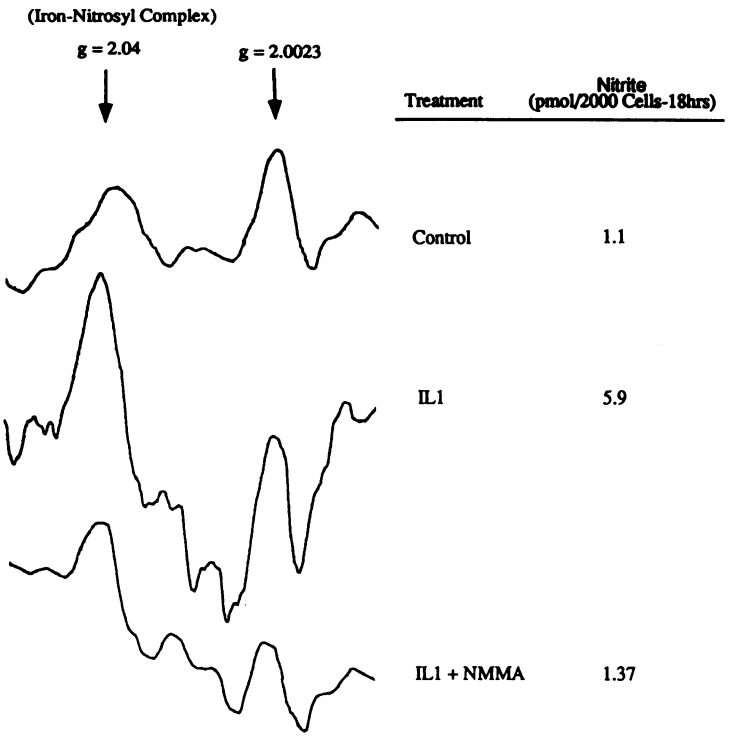

Figure 6. Effects of IL-1β and NMMA on EPR-detectable iron-nitrosyl complex and nitrite formation by Rin-m5F cells. Rin-m5F cells (~ 15×10^6 cells/3 ml) were treated for 18 h at 37°C in complete CMRL 1066 tissue culture media, supplemented with or without 5 U/ml IL-1β or 0.5 mM NMMA as indicated. The cells and supernatant were isolated by centrifugation and frozen at −70°C. EPR spectroscopy was performed on the cells at 77 K as described under Methods. The formation of nitrite in the culture supernatant was determined as described under Methods. The results are representative of three individual experiments containing at least 15×10^6 Rin-m5F cells per condition. The IL-1β–induced g = 2.04 iron-nitrosyl feature, and the g = 2.0023 delocalized electron feature are as indicated by the arrows.

aconitase activity of dispersed islet cells, and Rin-m5F cells was investigated. Pretreatment of dispersed islet cells for 18 h with IL-1β results in a 77% inhibition of aconitase activity that is completely prevented by pretreatment with NMMA in addition to IL-1β (Table 1 A). IL-1β pretreatment of Rin-m5F cells results in a 35% inhibition of aconitase activity, which is also prevented by pretreatment with NMMA in addition to IL-1β (Table 1 B).

Discussion

It has recently been demonstrated that IL-1β–induced inhibition of glucose-stimulated insulin secretion by pancreatic islets is dependent on protein synthesis and the metabolism of L-arginine to nitric oxide and L-citrulline (9, 10, 22). Nitric oxide is believed to be produced by the inducible isoform of nitric oxide synthase, and it appears to function as an effector molecule mediating IL-1β–induced islet dysfunction (10, 22). The pancreatic islet is a complex microorgan composed primarily of insulin-secreting β-cells (60–65%) and glucagon-secreting α-cells (25–30%), as well as significantly lower levels of somatostatin secreting δ-cells (23). Also present in the islet are circulating macrophages (~ 5–10/islet) and other nonendocrine cells including fibroblasts, dendritic, and endothelial cells (24). Since macrophages express the cytokine-inducible isoform of nitric oxide synthase, and other accompanying cells in the islet may also express this isoform of nitric oxide synthase, the endocrine cell type(s) in the islet responsible for the production of nitric oxide was investigated.

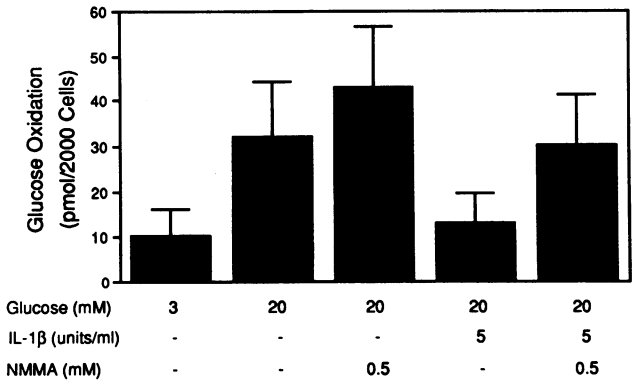

Figure 7. Effects of NMMA on IL-1β–induced inhibition of glucose oxidation by FACS®-purified β-cells. β-cells purified by FACS® cell sorting were pretreated for 18 h at 37°C in complete CMRL 1066 tissue culture media or complete CMRL 1066 containing 5 U/ml IL-1β, 0.5 mM NMMA, or both IL-1β and NMMA. The β-cells were isolated and the oxidation of [^{14}C]D-glucose to $^{14}CO_2$ was measured as described under Methods. Results are the mean±SEM of four individual experiments.

Table I. Effects of IL-1β and NMMA on Mitochondrial Aconitase Activity of Rin-m5F Cells and Islet Cells

Pretreatment 18 h at 37°C	Aconitase activity	Percent of control
	nmol/min per mg	
A Islet cells		
Control	7.2±0.2	100
NMMA (0.5 mM)	8.6±0.4	119
IL-1β (5 U/ml)	1.7±0.1*	23
IL-1β + NMMA	9.0±0.3	125
B Rin-m5F cells		
Control	21.0±1.7	100
NMMA (0.5 mM)	19.8±1.4	94
IL-1β (5 U/ml)	13.8±1.1*	65
IL-1β + NMMA	22.3±3.1	106

In this report, evidence is presented which indicates that IL-1β induces nitric oxide formation by the β-cell of the pancreatic islet, and that IL-1β does not induce the formation of nitric oxide by purified α-cells. Pretreatment of purified β-cells with IL-1β results in an inhibition of glucose-stimulated insulin secretion, which is prevented by the nitric oxide synthase inhibitor NMMA. IL-1β also induces the accumulation of cGMP by purified β-cells, which is blocked by pretreatment with NMMA. IL-1β does not appear to induce the formation of cGMP by purified α-cells obtained by FACS® purification. These data suggest that IL-1β–induced inhibition of insulin secretion by purified β-cells is dependent on the activity of nitric oxide synthase, and that IL-1β induces the formation of nitric oxide by β-cells. It is possible that IL-1β also induces the formation of nitric oxide by α-cells, but the α-cell may contain low levels of soluble guanylate cyclase, making changes in cGMP a poor indicator of nitric oxide production. However, this interpretation is inconsistent with the absence of an inhibitory effect of IL-1β on α-cell oxidation of 20 mM D-glucose.

Purification of β-cells from a dispersed heterogeneous population of islet cells by fluorescence activated cell sorting is based on both the fluorescence of endogenous FAD, as well as the larger diameter of the β-cell in comparison to the α-cell. It is possible that the parameters used for β-cell purification also copurify macrophages, and that the formation of nitric oxide by the purified β-cell population may be the result of macrophages or other nonendocrine cells. To address this possibility, the effect of IL-1β on nitric oxide production by the insulinoma cell line Rin-m5F, which represents a homogeneous population of β-cells, was examined. IL-1β induces the formation of nitric oxide by Rin-m5F cells as demonstrated by (*a*) the accumulation of cGMP; (*b*) EPR detectable iron-dinitrosyl complex formation; and (*c*) the ability of NMMA to block the formation of both cGMP and iron-dinitrosyl complexes. Furthermore, Drapier et al. (30) have presented evidence that indicates that IL-1β does not induce the formation of nitric oxide by macrophages. These results establish that IL-1β induces the formation of nitric oxide by both FACS®-purified β-cells, as well as the insulinoma cell line Rin-m5F, and that the formation of nitric oxide is probably not caused by the actions of IL-1β on macrophages or other cell types copurified by FACS® cell sorting.

IL-1β is believed to induce the expression of nitric oxide synthase by isolated islets. We have shown previously that cycloheximide blocks IL-1β–induced inhibition of glucose-stimulated insulin secretion and nitric oxide formation by isolated islets in a concentration-dependent fashion (8, 22). In the current study, cycloheximide is shown to block nitric oxide formation by Rin-m5F cells in a concentration-dependent manner that is similar to the effects of this protein synthesis inhibitor on nitric oxide production by islets (22). These results suggest that IL-1β induces the expression of nitric oxide synthase by β-cells of the islet and provide further evidence for an effector role of nitric oxide in mediating IL-1β–induced inhibition of insulin secretion by purified β-cells.

The cellular mechanism by which IL-1β–induced nitric oxide formation mediates an impaired insulin secretory response is unknown. The formation of an IL-1β–induced iron-dinitrosyl complex by Rin-m5F cells (Fig. 6) suggests that nitric oxide mediates the destruction of iron-sulfur centers resulting in the inhibition of iron-containing enzymes. Authentic nitric oxide has been shown to inhibit mitochondrial electron transport at

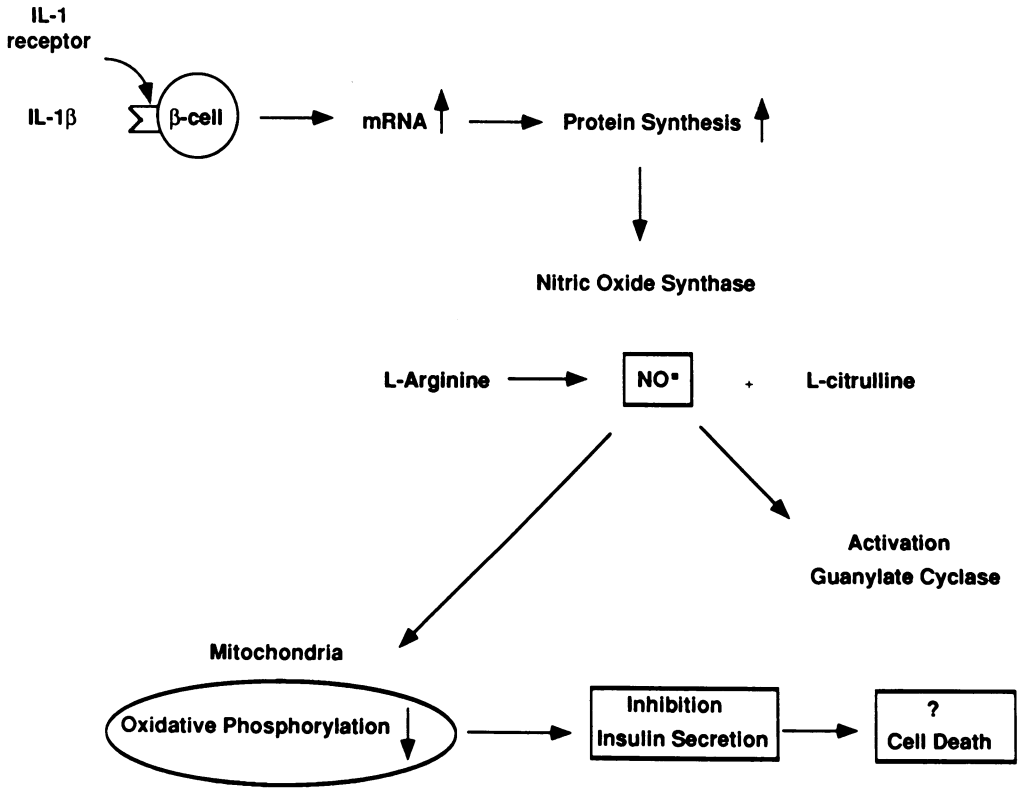

Figure 8. Proposed model for IL-1β–mediated β-cell dysfunction and destruction.

complexes 1 and 2, as well as aconitase activity in rat hepatocytes (31). Furthermore, treatment of macrophages with tumor necrosis factor and LPS induce the formation of an iron-dinitrosyl complex (32, 33, 34) and also results in the inhibition of mitochondrial electron transport and aconitase activity (35, 36). The common feature of these enzymes is that they contain iron-sulfur clusters that are necessary for their enzymatic activity. Pretreatment of dispersed islet cells with IL-1β results in a 77% reduction in aconitase activity that is prevented by NMMA (Table I). IL-1β pretreatment of Rin-m5F cells also results in an inhibition of aconitase activity (35%) that is prevented by NMMA. These results are consistent with those of Welsh et al. (37, 38), who have recently demonstrated that IL-1β induces the formation of nitric oxide and inhibits aconitase activity of both rat and mouse islets. Since pretreatment of Rin-m5F cells with IL-1β induces the formation of iron-nitrosyl complexes and results in an inhibition of aconitase activity, and also induces an inhibition of glucose oxidation by purified β-cells and dispersed islet cells, it is proposed that IL-1β mediates its inhibitory effects on insulin secretion from β-cells by the induction of nitric oxide synthase resulting in the formation of nitric oxide. The free radical, nitric oxide, induces an impairment of mitochondrial oxidative metabolism (specifically aconitase, and possibly electron transport) that is believed to be a consequence of the destruction of iron-sulfur centers.

A model describing the cellular mechanism by which IL-1β may induce β-cell dysfunction and destruction is proposed in Fig. 8. In this model, IL-1β binds to a β-cell specific receptor. Evidence for IL-1β receptors has been demonstrated in both insulinoma cell lines Rin-m5F and HIT (39, 40). The binding of IL-1β to its receptor triggers a signal transduction process that results in the induction of mRNA transcription. Hughes et al. (8, 37, 41) have previously shown that the transcriptional inhibitor, actinomycin D, prevents IL-1β–induced inhibition of insulin secretion, and that IL-1β induces the expression of the c-fos proto-oncogene in purified β-cells. IL-1β–induced gene transcription is followed by an induction of protein translation, which is believed to include the synthesis of nitric oxide synthase as well as possible autoantigens (8, 37, 42). Nitric oxide is produced by the mixed functional oxidation of L-arginine by nitric oxide synthase. It is proposed that nitric oxide functions as an effector molecule mediating the destruction of iron-sulfur clusters of mitochondrial iron containing enzymes; e.g., aconitase. Mitochondrial impairment by nitric oxide would result in a reduced level of cellular ATP (22), and an inhibition of insulin secretion. It is proposed that β-cell specific death is caused by nitric oxide–mediated inhibition of mitochondrial function, as well as the inhibition of DNA synthesis by nitric oxide (43).

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